

**TTK IN DIAGNOSIS AND AS A THERAPEUTIC TARGET IN CANCER****CROSS-REFERENCE TO RELATED APPLICATION**

This application claims the benefit of U.S. provisional application serial no.  
5 60/289,813, filed February 21, 2001, which application is hereby incorporated by reference.

**FIELD OF THE INVENTION**

The field of the present invention relates to disease diagnosis and treatment of cancer  
and identification of anti-cancer agents.

**BACKGROUND OF THE INVENTION**

Mitotic checkpoint genes have become widely studied for their roles in development as  
well as for their potential role in disease such as cancer. The mitotic checkpoint involves a  
number of different mechanisms to ensure proper cellular division. For example, the spindle  
15 assembly checkpoint modulates the timing of anaphase initiation in response to the improper  
alignment of chromosomes at the metaphase plate. If defects are detected, a signal is  
transduced to halt further progression of the cell cycle until correct bipolar attachment to the  
spindle is achieved. Initially identified in budding yeast, several mammalian spindle  
checkpoint-associated proteins have recently been identified and partially characterized. These  
20 proteins associate with all active human centromeres, including neocentromeres, in the early  
stages of mitosis prior to the commencement of anaphase. The proteins associated with the  
checkpoint protein complex (BUB1, BUBR1, BUB3, MAD2), the anaphase-promoting  
complex (Tsg24, p55CDC), and other proteins associated with mitotic checkpoint control  
(ERK1, 3F3/2 epitope, hZW10), were found to specifically associate with only the active  
25 centromere, suggesting that only active centromeres participate in the spindle checkpoint.  
Saffery R et al., *Hum Genet.* 107:376-84 (2000).

Tyrosine threonine kinase (TTK), a protein kinase, phosphorylates serine, threonine,  
and tyrosine hydroxyamino acids (Mills et al., *Biol. Chem.* 267:16000-6 (1992)). The kinases  
most closely related to TTK include SPK1 serine, threonine, and tyrosine kinase, the Pim1,  
30 PBS2, and CDC2 serine/threonine kinases, and the TIK kinase (Mills et al. *J. Biol. Chem.*

267:16000-6 (1992)). The nucleotide and amino acid sequences of human TTK are provided at, for example, GenBank Accession No. M86699. Expression of TTK is markedly reduced or absent in resting cells and in tissues with a low proliferative index (Hogg et al. *Oncogene* 9:89-96 (1994)). TTK mRNA is expressed in human testis, thymus, bone marrow, and other tissues that contain a large number of proliferating cells and is not detected in tissues that contain few or no dividing cells. TTK expression was detected in several rapidly proliferating cells lines, including various cancer cell lines. TTK expression was also detected and in samples tissue samples from two patients with malignant ovarian cancer (Mills et al., *ibid.*; Schmandt et al. *J. Immunol.* 152:96-105 (1994)). TTK expression is correlated with cell proliferation, and plays a role in cell cycle control (Hogg et al., *ibid.*). Very low levels of TTK mRNA and protein are present in starved cells. When cells are induced to enter the cell cycle, levels of TTK mRNA, protein, and kinase activity increase at the G1/S phase of the cell cycle and peak in G2/M. TTK mRNA levels, as well as kinase activity, drop sharply in early G1, whereas protein levels are largely maintained. TTK is a human homologue of the *S. cerevesiae* kinase mps1 and the *S. pombe* protein mph1, both of which are involved in cell cycle spindle assembly checkpoint, thus indicating that TTK is a spindle checkpoint gene (see, e.g., Cahill et al. *Genomics* 58:181-7 (1999)).

Although mitotic checkpoint impairment has been detected in human cancers (e.g., such impairment is present in about 40% of human lung cancer cell lines) mutations in the MAD mitotic checkpoint genes and the BUB gene family are infrequent. Haruki N et al., *Cancer Lett.* 162:201-205 (2001); Mimori K et al., *Oncol Rep.* 8:39-42 (2001); Cahill et al., *ibid.*). There is thus a need for identification of mitotic checkpoint genes that have a role in human cancers, as they can serve as informative diagnostic and/or prognostic indicators, and therapeutic targets.

#### SUMMARY OF THE INVENTION

The present invention provides methods for identification of cancerous cells by detection of expression levels of TTK, as well as diagnostic, prognostic and therapeutic methods that take advantage of the differential expression of these genes in mammalian cancer. Such methods can be useful in determining the ability of a subject to respond to a particular therapy, e.g., as the basis of rational therapy. In addition, the invention provides assays for identifying pharmaceuticals that modulate activity of these genes in cancers in which these

genes are involved, as well as methods of inhibiting tumor growth by inhibiting activity of TTK.

In a first embodiment, the present invention provides a method for identifying TTK levels in a sample of a subject suspected of having cancer (*e.g.*, a lung, colon, prostate or breast tissue biopsy) comprising quantifying the level of TTK in the sample. The identification of increased levels of TTK in the sample provides an indication of impairment of the cell cycle checkpoint in the sampled cells.

In another embodiment, the invention provides a method for determining the characteristics of a malignant or pre-malignant growth comprising determining (either qualitatively or quantitatively) the level of TTK in the cells of the growth, and comparing levels with known levels in various stages of cancer and/or normal tissue. For example, to determine the characteristics of a particular subject's colon cancer, a sample of the cancer may be removed, the levels of TTK in the cancer determined, and the levels compared to normal tissue and/or levels in various stage colon cancers derived from the same cell type. The levels of TTK identified in the sample can thus be indicative of various characteristics of the malignant or pre-malignant growth, as determined by the characteristics of known tissue and cancers. The TTK levels can be compared directly to the levels in other single samples, or may be compared to a standard that is derived from the data of multiple samples.

In another embodiment, the TTK levels of a sample can be used as one index for determining the appropriate therapeutic intervention for a subject with a malignant or pre-malignant growth. Highly increased levels of TTK, for example, can be indicative of the need for more aggressive therapy, as it is indicative of a later stage cancer. Alternatively, the level of TTK expression may be indicative of the responsiveness of a subject to a particular pharmaceutical, and in particular to a therapeutic intervention that affects the cancer via the mitotic checkpoint.

In another embodiment, the invention features a method for identifying agents for inhibiting growth of a tumor, particular by a breast or colon tumor, by contacting a cell expressing TTK with a candidate agent, and assessing the effect of the agent upon TTK activity.

Accordingly, in one aspect the invention features a method of diagnosing cancer in a subject, the method comprising detection of TTK polynucleotide or polypeptide in a test sample obtained from a subject so as to determine a level of expression of the gene product;

and comparing the level of expression of the TTK in the test sample to a level of expression in a normal cell corresponding to the same tissue; wherein detection of an expression level of TTK in the test sample that is significantly increased from the level of expression in a normal cell indicates that the test cell is cancerous. In specific embodiments, the cancer is other than ovarian cancer, with colon cancer and breast cancer being of particular interest.

In another aspect, the invention features a method for determining the prognosis of a cancerous disease in a subject, the method comprising detecting expression of TTK in a test cell from the subject; and comparing a level of expression of TTK in the test cell with a level of TTK expression in a control cell; wherein the level of expression of TTK in the test cell relative to the level of expression in the control cell is indicative of the prognosis of the cancerous disease. For example, where the control cell is a normal cell, an elevated level of TTK expression in the test cell relative to the normal cell is indicative of the continued presence of cancerous cells in the subject and thus a relatively poorer prognosis than where the level of TTK expression in the test cell is at a level comparable to that found in an normal (non-cancer) cell. In specific embodiments, progress of a cancer other than ovarian cancer is of particular interest, especially colon and breast cancer.

In another aspect, the invention features a method for inhibiting growth of a cancerous cell comprising introducing into a cell an antisense polynucleotide for inhibition of TTK expression, wherein inhibition of TTK expression inhibits replication of the cancerous cell.

In still another aspect, the invention features a method for assessing the tumor burden of a subject, the method comprising detecting a level of TTK expression in a test sample from a subject, the test sample suspected of comprising increased TTK expression; wherein detection of the level of TTK expression in the test sample is indicative of the tumor burden in the subject, with an increased level of TTK expression in the test sample relative to a control non-cancer cell indicates the presence of a tumor in the subject.

In yet another aspect, the invention features a method of identifying an agent having anti-TTK activity, the method comprising contacting a cancerous cell displaying elevated expression of TTK with a candidate agent; and determining the effect of the candidate agent on TTK activity; wherein a decrease in TTK activity indicates that the agent has anti-TTK activity. In specific embodiments, TTK activity is detected by detecting TTK expression or by detecting a biological activity of TTK

In yet another aspect, the invention features an assay for identifying a candidate agent that inhibits growth of a cancerous cell, comprising contacting a cell expressing TTK polypeptide with a candidate agent; and detecting activity of the TTK polypeptide, comparing the activity of the TTK polypeptide in the cell in the presence of the candidate agent to activity of a TTK polypeptide in a cell in the absence of the candidate agent; wherein reduction of TTK activity in the presence of the candidate agent relative to TTK activity in the absence of the candidate agent indicates that the candidate agent reduces TTK activity and inhibits growth of a cancerous cell.

A primary object of the invention is to exploit TTK as a therapeutic target, e.g. by identifying candidate agents that modulate, usually that decrease, TTK activity in a target cell in order to, for example, inhibit cell growth.

An object of the present invention is to inhibit tumor growth by inhibition of activity of a mitotic checkpoint gene product, particularly through inhibition of TTK activity in the target tumor cell.

Another object of the invention is to facilitate rational cancer therapy. For example, where the cancer in the subject is associated with increased TTK activity levels, a therapeutic agent is selected accordingly so as to facilitate reduction of TTK activity levels.

Another object of the present invention is to design clinical trials based on levels of TTK expression in a cancer, and more particularly to design clinical trials based on TTK expression in combination with other patient attributes.

Yet another object of the invention is to identify the association of TTK expression and intervention attributes that yield efficacious changes in selected disease progression measures.

An advantage of the invention is the ability to project disease progression based on expression of TTK in a malignant or pre-malignant growth.

Another advantage of the present invention is that it allows a more systematic approach for intervention of a cancerous disease based upon objective indicia.

These and other objects, advantages, and features of the invention will become apparent to those persons skilled in the art upon reading the details of the methods as more fully described below.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a bar graph illustrating expression of TTK in various normal tissue types as detected by PCR.

FIG. 2 is a bar graph illustrating expression of TTK in various tumor cell lines as detected by PCR.

FIGS. 3-6 are graphs illustrating expression profiles for IGF2, MAPKAPK2, TTK, and MARCKS in patients with colorectal carcinoma.

FIGS. 7 and 8 are graphs illustrating growth suppression of MDA-MB-231 cells following antisense suppression of TTK expression.

FIG. 9 is a graph illustrating growth suppression of SW620 cells following antisense suppression of TTK expression.

FIG. 10 is a graph illustrating suppression of colony formation of SW620 cells in soft agar following antisense suppression of TTK expression.

FIG. 11 is a graph illustrating that antisense suppression of TTK has no detectable effect on normal immortal fibroblasts.

FIG. 12 is a bar graph illustrating induction of cell death upon depletion of TTK from SW620 cells.

## DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Before the present invention is described, it is to be understood that this invention is not limited to particular methodologies described, and as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range

includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the agent" includes reference to one or more agents and equivalents thereof known to those skilled in the art, and so forth.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

## DEFINITIONS

The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric forms of nucleotides of any length, either ribonucleotides or deoxynucleotides. Thus, these terms include, but are not limited to, single-, double-, or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. These terms further include, but are not limited to, mRNA or cDNA that comprise intronic sequences (see, *e.g.*, Niwa et al. (1999) *Cell* 99(7):691-702). The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidites and thus can be an oligodeoxynucleoside phosphoramidate or a

mixed phosphoramidate-phosphodiester oligomer. Peyrottes et al. (1996) *Nucl. Acids Res.* 24:1841-1848; Chaturvedi et al. (1996) *Nucl. Acids Res.* 24:2318-2323. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars, and linking groups such as fluororibose and thioate, and nucleotide

5 branches. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling

10 components, other polynucleotides, or a solid support.

The terms "polypeptide" and "protein", used interchangeably herein, refer to a polymeric form of amino acids of any length, which can include coded and non-coded amino acids, chemically or biochemically modified or derivatized amino acids, and polypeptides having modified peptide backbones. The term includes fusion proteins, including, but not

15 limited to, fusion proteins with a heterologous amino acid sequence, fusions with heterologous and homologous leader sequences, with or without N-terminal methionine residues; immunologically tagged proteins; and the like.

As used herein "TTK polynucleotide" and "TTK polypeptide" encompass polynucleotides and polypeptides having sequence similarity or sequence identity to the human

20 TTK (having GenBank accession number M86699; SEQ ID NO:13 and 14), or the *S. cerevisiae* kinase mps1 gene and gene products (SEQ ID NO:29 and 30), the *S. pombe* protein mph1 gene and gene products (SEQ ID NO:31 and 32), and other genes and gene products related to TTK, such as SPK1 (SEQ ID NO:15 and 16), Pim1 (SEQ ID NO:17 and 18), PBS2 (SEQ ID NO:19 and 20), CDC2 (SEQ ID NO:21 and 22), and TIK (SEQ ID NO:23 and 24) of

25 at least about 65%, preferably at least about 80%, more preferably at least about 85%, and can be about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more. Sequence similarity and sequence identity are calculated based on a reference sequence, which may be a subset of a larger sequence, such as a conserved motif, coding region, flanking region, *etc.* A reference sequence will usually be at least about 18 nt long, more usually at least about 30 nt

30 long, and may extend to the complete sequence that is being compared. In general, percent sequence identity is calculated by counting the number of residue matches (*e.g.*, nucleotide residue or amino acid residue) between the query and test sequence and dividing total number



of matches by the number of residues of the individual sequences found in the region of strongest alignment. Thus, where 10 residues of an 11 residue query sequence matches a test sequence, the percent identity above would be 10 divided by 11, or approximately, 90.9%. Algorithms for computer-based sequence analysis are known in the art, such as BLAST (see, 5 *e.g.*, Altschul et al., *J. Mol. Biol.*, 215:403-10 (1990)), particularly the Smith-Waterman homology search algorithm as implemented in MPSRCH program (Oxford Molecular). For the purposes of this invention, a preferred method of calculating percent identity is the Smith-Waterman algorithm, using the following. Global DNA sequence identity must be greater than 65% as determined by the Smith-Waterman homology search algorithm as implemented in 10 MPSRCH program (Oxford Molecular) using an affine gap search with the following search parameters: gap open penalty, 12; and gap extension penalty, 1. The human TTK cDNA is represented by the polynucleotide sequence of SEQ ID NO:13 and the human TTK polypeptide is represented by the sequence of SEQ ID NO:14.

“Antisense polynucleotide” or “antisense oligonucleotide” are used interchangeably 15 herein to mean an unmodified or modified nucleic acid having a nucleotide sequence complementary to a given polynucleotide sequence (*e.g.*, a polynucleotide sequence encoding TTK) including polynucleotide sequences associated with the transcription or translation of the given polynucleotide sequence (*e.g.*, a promoter of a polynucleotide encoding TTK), where the antisense polynucleotide is capable of hybridizing to a TTK-encoding polynucleotide 20 sequence. Of particular interest are antisense polynucleotides capable of inhibiting transcription and/or translation of a TTK-encoding polynucleotide either *in vitro* or *in vivo*.

The term “cDNA” as used herein is intended to include all nucleic acids that share the arrangement of sequence elements found in native mature mRNA species, where sequence elements are exons (*e.g.*, sequences encoding open reading frames of the encoded polypeptide) 25 and 3’ and 5’ non-coding regions. Normally mRNA species have contiguous exons, with the intervening introns removed by nuclear RNA splicing to create a continuous open reading frame encoding TTK.

A “variant” as used in the context of a “variant polypeptide” refers to an amino acid sequence that is altered by one or more amino acids relative to a reference amino acid 30 sequence. The variant can have “conservative” changes, wherein a substituted amino acid has similar structural or chemical properties, *e.g.*, replacement of leucine with isoleucine. More rarely, a variant can have “nonconservative” changes, *e.g.*, replacement of a glycine with a

tryptophan. Similar minor variations can also include amino acid deletions or insertions, or both. Guidance in determining which and how many amino acid residues may be substituted, inserted, or deleted without abolishing biological or immunological activity can be found using computer programs well known in the art, for example, DNASTar software.

5       A "deletion" is defined as a change in either amino acid or nucleotide sequence in which one or more amino acid or nucleotide residues, respectively, are absent as compared to reference amino acid sequence or nucleotide sequence. Deletions can be of any length, but are preferably approximately 50, 20, 15, 10, 5 or 3 amino acids or nucleotides in length.

10       An "insertion" or "addition" is that change in an amino acid or nucleotide sequence which has resulted in the addition of one or more amino acid or nucleotide residues, respectively, as compared to a reference amino acid sequence or nucleotide sequence. Insertions or additions can be of any length, but are preferably approximately 50, 20, 15, 10, 5 or 3 amino acids or nucleotides in length.

15       A "substitution" results from the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively, as compared to a reference amino acid sequence or nucleotide sequence. Substitutions can be of any length, but are preferably approximately 50, 20, 15, 10, 5 or 3 amino acids or nucleotides in length.

The terms "single nucleotide polymorphism" and "SNP" refer to polymorphisms of a single base change relative to a reference sequence.

20       The term "biologically active" refers to gene product, usually a polypeptide, having structural, regulatory, or biochemical functions of a naturally occurring gene product, *e.g.*, protein. "Immunologically active" defines the capability of the natural, recombinant, or synthetic polypeptide, or any oligopeptide thereof, to elicit a specific immune response in appropriate animals or cells and to bind with specific antibodies.

25       The term "derivative" as used herein refers to the chemical modification of a nucleic acid or amino acid sequence relative to a reference nucleic acid or amino acid sequence. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group. A nucleic acid derivative generally encodes a polypeptide which retains essential biological characteristics of the polypeptide encoded by the reference nucleic acid  
30       (*e.g.*, the "parent" molecule).

As used herein the term "isolated" is meant to describe a compound of interest (*e.g.*, either a polynucleotide or a polypeptide) that is in an environment different from that in which

the compound naturally occurs. "Isolated" is meant to include compounds that are within samples that are substantially enriched for the compound of interest and/or in which the compound of interest is partially or substantially purified.

As used herein, the term "substantially purified" refers to a compound (*e.g.*, either a polynucleotide or a polypeptide) that is removed from its natural environment and is at least 60% free, preferably 75% free, and most preferably 90% free from other components with which it is naturally associated.

"Stringency" typically occurs in a range from about  $T_m - 5^\circ\text{C}$  ( $5^\circ\text{C}$  below the  $T_m$  of the probe or antibody) to about  $20^\circ\text{C}$  to  $25^\circ\text{C}$  below  $T_m$ . As will be understood by those of skill in the art, a stringency hybridization can be used to identify or detect identical polynucleotide sequences or to identify or detect similar or related polynucleotide sequences.

The term "hybridization" as used herein shall include "any process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs, Dictionary of Biotechnology, Stockton Press, New York NY (1994)). Amplification as carried out in the polymerase chain reaction technologies is described in Dieffenbach et al., PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY (1995).

The term "transformation" as used herein refers to a permanent or transient genetic change, induced in a cell following incorporation of new DNA (*i.e.*, DNA exogenous to the cell). Genetic change can be accomplished either by incorporation of the new DNA into the genome of the host cell, or by transient or stable maintenance of the new DNA as an episomal element. Where the cell is a mammalian cell, a permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell.

The term "construct" as used herein refers to a recombinant nucleic acid, generally recombinant DNA, that has been generated for the purpose of the expression of a specific nucleotide sequence(s), or is to be used in the construction of other recombinant nucleotide sequences.

As used herein, the term "differentially expressed" generally refers to a polynucleotide that is expressed at levels in a test cell that differ significantly from levels in a reference cell, *e.g.*, mRNA is found at levels at least about 25%, at least about 50% to about 75%, at least about 90% increased or decreased, generally at least about 1.2-fold, at least about 1.5-fold, at least about 2-fold, at least about 5-fold, at least about 10-fold, or at least about 50-fold or more increased or decreased in a cancerous cell when compared with a cell of the same type that is

not cancerous. The comparison can be made between two tissues, for example, if one is using in situ hybridization or another assay method that allows some degree of discrimination among cell types in the tissue. The comparison may also be made between cells removed from their tissue source. "Differential expression" refers to both quantitative, as well as qualitative, differences in the genes' temporal and/or cellular expression patterns among, for example, normal and neoplastic tumor cells, and/or among tumor cells which have undergone different tumor progression events.

The terms "correspond to" or "represents" as used in, for example, the phrase "polynucleotide corresponds to a differentially expressed gene" are used to refer to the relationship between a given polynucleotide and the gene from which the polynucleotide sequence is derived (*e.g.*, a polynucleotide that is derived from a coding region of the gene, a splice variant of the gene, an exon, and the like) or to which the polynucleotide hybridizes to under stringer conditions.

"Differentially expressed polynucleotide" as used herein refers to a nucleic acid molecule (RNA or DNA) comprising a sequence that represents or corresponds to a differentially expressed gene, *e.g.*, the differentially expressed polynucleotide comprises a sequence (*e.g.*, an open reading frame encoding a gene product; a non-coding sequence) that uniquely identifies a differentially expressed gene so that detection of the differentially expressed polynucleotide in a sample is correlated with the presence of a differentially expressed gene in a sample. "Differentially expressed polynucleotides" is also meant to encompass fragments of the disclosed polynucleotides, *e.g.*, fragments retaining biological activity, as well as nucleic acids homologous, substantially similar, or substantially identical (*e.g.*, having about 90% sequence identity) to the disclosed polynucleotides.

"Diagnosis" as used herein generally includes determination of a subject's susceptibility to a disease or disorder, determination as to whether a subject is presently affected by a disease or disorder, prognosis of a subject affected by a disease or disorder (*e.g.*, identification of pre-metastatic or metastatic cancerous states, stages of cancer, or responsiveness of cancer to therapy), and therametrics (*e.g.*, monitoring a subject's condition to provide information as to the effect or efficacy of therapy).

As used herein, the term "a polypeptide associated with cancer" (*e.g.*, as in polypeptide associated with colon cancer) refers to a polypeptide that is present at relatively higher or lower levels in a cancer cell relative to a normal cell of the same type.

The term "biological sample" encompasses a variety of sample types obtained from an organism and can be used in a diagnostic or monitoring assay. The term encompasses blood and other liquid samples of biological origin, solid tissue samples, such as a biopsy specimen or tissue cultures or cells derived therefrom and the progeny thereof. The term encompasses samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components. The term encompasses a clinical sample, and also includes cells in cell culture, cell supernatants, cell lysates, serum, plasma, biological fluids, and tissue samples.

The terms "treatment", "treating", "treat" and the like are used herein to generally refer to obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete stabilization or cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particularly a human, and includes: (a) preventing the disease or symptom from occurring in a subject which may be predisposed to the disease or symptom but has not yet been diagnosed as having it; (b) inhibiting the disease symptom, *i.e.*, arresting its development; or relieving the disease symptom, *i.e.*, causing regression of the disease or symptom. Thus "treatment of cancer" thus encompasses one or more of inhibition of cellular proliferation, inhibition of metastasis, and the like.

The terms "individual," "subject," "host," and "patient," used interchangeably herein and refer to any mammalian subject for whom diagnosis, treatment, or therapy is desired, particularly humans. Other subjects may include cattle, dogs, cats, guinea pigs, rabbits, rats, mice, horses, and so on.

The phrase "specific binding pair" as used herein comprises a specific binding member and a binding partner which have a particular specificity for each other and which bind to each other in preference to other molecules under stringent conditions. Examples of specific binding pairs are antigens and antibodies, molecules and receptors and complementary nucleotide sequences. Other examples of binding pairs will be apparent to one skilled in the art upon reading the present disclosure. Further, the term "specific binding pair" is also applicable where either or both of the specific binding member and the binding partner comprise a part of a larger molecule. In embodiments in which the specific binding pair are

nucleic acid sequences, they are preferably between 10 to 200 nucleotides long, more preferably greater than 15 to 100 nucleotides long.

By "antibody" is meant an immunoglobulin protein which is capable of binding an antigen. Antibody as used herein is meant to include the entire antibody as well as any  
 5 antibody fragments (*e.g.*, F(ab')<sub>2</sub>, Fab', Fab, Fv) capable of binding the epitope, antigen, or antigenic fragment of interest.

Antibodies of the invention are immunoreactive or immunospecific for and therefore specifically and selectively bind to a protein of interest, *e.g.*, human TTK protein. Antibodies which are immunoreactive and immunospecific for human TTK are preferred. Antibodies for  
 10 human TTK are preferably immunospecific -- *i.e.*, not substantially cross-reactive with related materials, although they may recognize TTK homologs across species. The term "antibody" encompasses all types of antibodies (*e.g.*, monoclonal and polyclonal).

By "binds specifically" is meant high avidity and/or high affinity binding of an antibody to a specific polypeptide, *e.g.*, epitope of a TTK protein. Antibody binding to its  
 15 epitope on this specific polypeptide is stronger than binding of the same antibody to any other epitope, particularly those which may be present in molecules in association with, or in the same sample, as the specific polypeptide of interest. Antibodies which bind specifically to a polypeptide of interest may be capable of binding other polypeptides at a weak, yet detectable, level (*e.g.*, 10% or less of the binding shown to the polypeptide of interest). Such weak  
 20 binding, or background binding, is readily discernible from the specific antibody binding to the compound or polypeptide of interest, *e.g.*, by use of appropriate controls.

The terms "cancer", "neoplasm", "tumor", and the like are used interchangeably herein to refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation.  
 25 In general, cells of interest for detection or treatment in the present application include pre-malignant (*e.g.*, benign hyperplastic), malignant, metastatic, and non-metastatic cells.

"TTK activity" as used herein refers to activity of the TTK polypeptide in phosphorylation of a recipient substrate.

"Modulation of TTK activity" as used herein refers to an increase or decrease in  
 30 TTK activity that can be a result of, for example, interaction of an agent with a TTK polypeptide (*e.g.*, reversible or irreversible binding of an inhibitory agent so as to interfere with TTK polypeptide interaction with a donor molecule or a recipient (acceptor) molecule in the

phosphorylation activity of TTK), inhibition of TTK transcription and/or translation (*e.g.*, through antisense interaction with the TTK gene or TTK transcript, through modulation of transcription factors that facilitate TTK expression), and the like. Modulation of TTK activity that results in a decrease of TTK activity is of particular interest in the invention. In this context, TTK activity can be decreased by an inhibitory agent at least 10%, 25%, 50%, 75%, 85%, 90%, up to 100% relative to TTK activity in the absence of an agent. TTK activity can be assessed by assaying enzymatic activity, by assessing TTK polypeptide levels, or by assessing TTK transcription levels. Comparisons of TTK activity can also be accomplished by comparing TTK activity assessed (either qualitatively or quantitatively) in a test sample to a standard TTK activity (*e.g.*, a level of TTK activity in the absence of an inhibitory agent or agonist, that is associated with a normal cell, a level of TTK activity of a cancerous cell of a selected tissue type, and the like).

#### Overview

Human TTK is a mitotic checkpoint gene which encodes an 857 amino acid protein that exhibits activity of a mixed specificity (tyr/thr) kinase. TTK is expressed in rapidly proliferating tissues such as testis and thymus. See, *e.g.*, Mills GB et al., *J Biol Chem.* 267:16000-6 (1992). The present invention is based upon the finding that TTK is differentially expressed in colon tumor cells relative to normal colon cells as detected by microarray analysis. Differential expression was confirmed in cell lines derived from various forms of cancer, indicating that the involvement of TTK in cancer as a more general mechanism. In addition, disruption of TTK function using antisense oligonucleotides to “knock-out” TTK message decreased proliferation, inhibited anchorage independent growth, and induced apoptosis of cancer cell lines, including a metastatic breast cancer cell line (MDA-MB-213) and a colorectal carcinoma cell line (SW620). These data indicate that TTK can be a therapeutic target for chemotherapy in cancers in which TTK is overexpressed.

The identification of the association of TTK with cancer, and the confirmation that inhibition of TTK activity (*e.g.*, by reducing TTK expression) serves as the basis for the materials and methods of the invention, such as are disclosed and discussed herein, for use in, for example, diagnosing cancer of a patient, particularly a cancer that is susceptible to treatment by decreasing activity of TTK. The invention also provides for planning and selection of appropriate therapeutic and/or prophylactic treatment, permitting streamlining of treatment by targeting those most likely to benefit. The invention also provides for treatment

of a cancer associated with aberrant TTK levels (*e.g.*, associated with overexpression or overproduction of TTK), *e.g.* by inhibition of gene product production (*e.g.*, decreasing levels of transcription and/or translation), by decreasing TTK activity (*e.g.*, by decreasing TTK gene product production (*e.g.*, at the level of transcription or translation) and/or by reducing one or more of TTK's kinase activities).

Various aspects of the invention will now be described in more detail.

#### Diagnostic Methods

In one aspect the invention is based on the discovery that TTK activity is present at higher levels in cancerous cells (particularly in colon cancer and breast cancer) than in normal cells of the same cell type. This discovery serves as the basis for identification of cancerous cells, as well as identification of tumors that are susceptible to therapy by inhibiting activity of TTK, *e.g.*, by inhibiting TTK expression at the level of transcription or translation or both, by inhibiting TTK activity, and the like.

TTK gene products *e.g.* TTK encoding mRNA or TTK polypeptides are of particular interest as markers (*e.g.*, in bodily fluids (such as blood) or in tissues) to detect the earliest changes along the carcinogenesis pathway (*e.g.*, to differentiate cancerous tissue from non-cancerous tissue) and/or to monitor the efficacy of various therapies and preventive interventions. For example, a relatively increased level of expression of TTK compared to normal cells or tissues of the same type can be indicative of a poorer prognosis, and therefore warrant more aggressive therapy (*e.g.*, chemo- or radio-therapy) for a patient or vice versa. The correlation of surrogate tumor specific features with response to treatment and outcome in patients can define prognostic indicators that allow the design of tailored therapy based on the molecular profile of the tumor. These therapies include antibody targeting, antagonists (*e.g.*, small molecules), and gene therapy. Determining TTK expression and comparison of a patient's profile with known expression in normal tissue and variants of the disease allows a determination of the best possible treatment for a patient, both in terms of specificity of treatment and in terms of comfort level of the patient. Surrogate tumor markers, such as polynucleotide expression, can also be used to better classify, and thus diagnose and treat, different forms and disease states of cancer. Two classifications widely used in oncology that can benefit from identification of TTK expression levels are staging of the cancerous disorder, and grading the nature of the cancerous tissue.



TTK polynucleotides, as well as their encoded gene products, can be useful to monitor patients having or susceptible to cancer to detect potentially malignant events at a molecular level before they are detectable at a gross morphological level. In addition, detection of TTK gene products can be useful as therametrics, *e.g.*, to assess the effectiveness of therapy by using the polynucleotides or their encoded gene products, to assess, for example, tumor burden in the patient before, during, and after therapy.

Furthermore, a polynucleotide identified as corresponding to a gene that is differentially expressed in, and thus is important for, one type of cancer can also have implications for development or risk of development of other types of cancer, *e.g.*, where a polynucleotide represents a gene differentially expressed across various cancer types. Thus, for example, expression of a polynucleotide corresponding to a gene that has clinical implications for metastatic colon cancer can also have clinical implications for stomach cancer or endometrial cancer.

In making a diagnosis, prognosis, risk assessment, or measurement of tumor burden based on the enzymatic activity of TTK or the expression levels of TTK polypeptide or TTK encoding polynucleotides, activity or expression levels may be compared to those of suitable cancerous or non-cancerous control samples. For example, a diagnosis of cancer can be made if TTK activity is increased at by 25%, 50%, 75%, 90%, up to 100%, or, alternatively by 5-fold, 10-fold, 50-fold, or more than 100-fold relative to a normal non-cancerous cell of the same tissue type.

Other gene products that are differentially expressed in cancerous cells relative to, for example, non-cancer cells of between cancer cells of differing malignant potential (*e.g.*, non-malignant tumor cells versus cells of high potential malignancy) can also be assayed in addition to TTK for differential expression in a test cell. Such exemplary gene products include, but are not necessarily limited to MAPKAP kinase 2 (SEQ ID. No. 33 and 34), MARCKS (SEQ ID NO:35 and 36) and/or IGF2 (SEQ ID NO:37 and 38).

Staging. Staging is a process used by physicians to describe how advanced the cancerous state is in a patient. Staging assists the physician in determining a prognosis, planning treatment and evaluating the results of such treatment. Staging systems vary with the types of cancer, but generally involve the following "TNM" system: the type of tumor, indicated by T; whether the cancer has metastasized to nearby lymph nodes, indicated by N; and whether the cancer has metastasized to more distant parts of the body, indicated by M.

Generally, if a cancer is only detectable in the area of the primary lesion without having spread to any lymph nodes it is called Stage I. If it has spread only to the closest lymph nodes, it is called Stage II. In Stage II, the cancer has generally spread to the lymph nodes in near proximity to the site of the primary lesion. Cancers that have spread to a distant part of the body, such as the liver, bone, brain or other site, are Stage IV, the most advanced stage.

The differential expression level of TTK can facilitate fine-tuning of the staging process by identifying markers for the aggressiveness of a cancer, *e.g.* the metastatic potential, as well as the presence in different areas of the body. Thus, a Stage II cancer with a large differential level of expression of TTK can signify a cancer with a high metastatic potential and can be used to change a borderline Stage II tumor to a Stage III tumor, justifying more aggressive therapy.

Grading of cancers. Grade is a term used to describe how closely a tumor resembles normal tissue of its same type. The microscopic appearance of a tumor is used to identify tumor grade based on parameters such as cell morphology, cellular organization, and other markers of differentiation. As a general rule, the grade of a tumor corresponds to its rate of growth or aggressiveness, with undifferentiated or high-grade tumors generally being more aggressive than well differentiated or low-grade tumors. The following guidelines are generally used for grading tumors: 1) GX Grade cannot be assessed; 2) G1 Well differentiated; G2 Moderately well differentiated; 3) G3 Poorly differentiated; 4) G4 Undifferentiated. TTK activity levels (*e.g.*, expression levels) can be especially valuable in determining the grade of the tumor, as they not only can aid in determining the differentiation status of the cells of a tumor, they can also identify factors other than differentiation that are valuable in determining the aggressiveness of a tumor, such as metastatic potential.

Detection of colon cancer. Polynucleotides and polypeptides corresponding to TTK can be used to detect colon cancer in a subject. Colorectal cancer is one of the most common neoplasms in humans and perhaps the most frequent form of hereditary neoplasia. Prevention and early detection are key factors in controlling and curing colorectal cancer. Colorectal cancer begins as polyps, which are small, benign growths of cells that form on the inner lining of the colon. Over a period of several years, some of these polyps accumulate additional mutations and become cancerous. Multiple familial colorectal cancer disorders have been identified, which are summarized as follows: 1) Familial adenomatous polyposis (FAP); 2) Gardner's syndrome; 3) Hereditary nonpolyposis colon cancer (HNPCC); and 4) Familial

colorectal cancer in Ashkenazi Jews. The expression of appropriate polypeptide and polynucleotides can be used in the diagnosis, prognosis and management of colorectal cancer. Detection of colon cancer can be determined using expression levels of TTK alone or in combination with the levels of expression of other genes differentially expressed in colon cancer. Determination of the aggressive nature and/or the metastatic potential of a colon cancer can be determined by comparing levels of TTK with a level associated with a normal cell, and comparing total levels of another sequence known to be differentially expressed, or otherwise be a marker of, cancerous tissue, *e.g.*, expression of p53, DCC, ras, FAP (see, *e.g.*, Fearon ER, *et al.*, *Cell* (1990) 61(5):759; Hamilton SR *et al.*, *Cancer* (1993) 72:957; Bodmer W, *et al.*, *Nat Genet.* (1994) 4(3):217; Fearon ER, *Ann N Y Acad Sci.* (1995) 768:101) or MAPKAP kinase 2 (SEQ ID. No. 33 and 34), MARCKS (SEQ ID NO:35 and 36) and/or IGF2 (SEQ ID NO:37 and 38). For example, development of colon cancer can be detected by examining the level of expression of a gene corresponding to a polynucleotides described herein to the levels of oncogenes (*e.g.* ras) or tumor suppressor genes (*e.g.* FAP or p53). Thus expression of specific marker polynucleotides can be used to discriminate between normal and cancerous colon tissue, to discriminate between colon cancers with different cells of origin, to discriminate between colon cancers with different potential metastatic rates, etc. For a review of markers of cancer, see, *e.g.*, Hanahan et al. (2000) *Cell* 100:57-70.

Detection of breast cancer. The majority of breast cancers are adenocarcinomas subtypes, which can be summarized as follows: 1) ductal carcinoma in situ (DCIS), including comedocarcinoma; 2) infiltrating (or invasive) ductal carcinoma (IDC); 3) lobular carcinoma in situ (LCIS); 4) infiltrating (or invasive) lobular carcinoma (ILC); 5) inflammatory breast cancer; 6) medullary carcinoma; 7) mucinous carcinoma; 8) Paget's disease of the nipple; 9) Phyllodes tumor; and 10) tubular carcinoma.

The expression levels of TTK can be used in the diagnosis and management of breast cancer, as well as to distinguish between types of breast cancer. Detection of breast cancer can be determined using expression levels of TTK, either alone or in combination with expression of other gene known to be differentially expressed in breast cancer. Determination of the aggressive nature and/or the metastatic potential of a breast cancer can also be determined by comparing levels of TTK and comparing levels of another sequence known to vary in cancerous tissue, *e.g.* ER expression. In addition, development of breast cancer can be detected by examining the ratio of expression of TTK to the levels of steroid hormones (*e.g.*,

testosterone or estrogen) or to other hormones (e.g., growth hormone, insulin). Thus expression of specific marker polynucleotides and polypeptides can be used to discriminate between normal and cancerous breast tissue, to discriminate between breast cancers with different cells of origin, to discriminate between breast cancers with different potential metastatic rates, etc.

#### Detection methods

A number of methods are known in the art for analyzing biological samples from individuals to determine whether the individual has increased expression of a TTK gene product (e.g., RNA or protein) by detecting the TTK gene product in a biological sample from that subject. As discussed above, the purpose of such analysis may be used for diagnosis, to detect the presence of an existing cancer, to help identify the type of cancer, to assist a physician in determining the severity or likely course of the cancer, and/or to optimize treatment of it. In specific non-limiting embodiments, the methods are useful for detecting cancer cells, facilitating diagnosis of cancer and the severity of a cancer (e.g., tumor grade, tumor burden, and the like) in a subject, facilitating a determination of the prognosis of a subject, and assessing the responsiveness of the subject to therapy (e.g., by providing a measure of therapeutic effect through, for example, assessing tumor burden during or following a chemotherapeutic regimen). In additional embodiments, the methods are useful for classification or stratification of cancer cells, e.g., for the purpose of selecting patients to be included in a clinical trial population, for selecting an appropriate therapy (e.g., selecting therapy according to an expression profile of the cancerous cells), and the like.

#### Kits

The detection methods can be provided as part of a kit. Thus, the invention further provides kits for detecting the presence and/or a level of TTK activity e.g., by detection of a TTK-encoding mRNA and/or a polypeptide encoded thereby or by measuring TTK activity, in a biological sample. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private individuals. The kits of the invention for detecting TTK polypeptide that is differentially expressed in cancer cells comprise a moiety that specifically binds the polypeptide, which may be a specific antibody. The kits of the invention for detecting a TTK-encoding polynucleotide that is differentially expressed in cancer cells comprise a moiety that specifically hybridizes to such a polynucleotide such as a primer. The kits of the invention for detecting TTK activity comprise

a recipient substrate capable of being phosphorylated by TTK, and a labeled donor substrate. The kits may optionally provide additional components that are useful in the procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, standards, instructions, and interpretive information.

5        Screening for TTK nucleic acid or polypeptide

Methods for detection of TTK activity include screening for the presence of TTK nucleic acid sequences representing an expressed TTK gene or alleles or variants thereof, and detecting the TTK polypeptide. The methods make use of biological samples from individuals that are suspected of contain the nucleic acid sequences or polypeptide. Examples of  
10        biological samples include blood, plasma, serum, tissue samples, tumor samples, saliva and urine.

Exemplary approaches for detecting TTK nucleic acid or polypeptides include:

(a) determining the presence of the polypeptide encoded by the TTK gene; (b) using a specific binding member capable of binding to a TTK nucleic acid sequence (e.g., a known  
15        complementary sequence), the specific binding member comprising a nucleic acid that hybridizes with the TTK sequence under stringent conditions (c) using a substance comprising an antibody domain with specificity for a TTK nucleic acid sequence or the polypeptide encoded by it, the specific binding member being labeled to allow detection of the specific binding member to its binding partner is detectable; (d) using PCR involving one or more  
20        primers to determine relative levels of TTK in a sample from a patient; and (e) using an assay for TTK activity, e.g., phosphorylation of a TTK substrate.

The determination of TTK levels can include both levels of normal TTK and/or variant forms of TTK. A variant form of the gene may contain one or more insertions, deletions, substitutions and/or additions of one or more nucleotides compared with the wild-type  
25        sequence which may or may not alter the gene function. Differences at the nucleic acid level are not necessarily reflected by a difference in the amino acid sequence of the encoded polypeptide due to the degeneracy of the genetic code. However, a mutation or other difference in a gene may result in a frame-shift or stop codon, which could seriously affect the nature of the polypeptide produced (if any), or a point mutation or gross mutational change to  
30        the encoded polypeptide, including insertion, deletion, substitution and/or addition of one or more amino acids or regions in the polypeptide.

A mutation in a promoter sequence or other regulatory region may alter (*e.g.*, reduce or enhance) expression from the gene or affect the processing or stability of the mRNA transcript.

There are various methods for detecting a particular nucleic acid sequence in a test sample. Tests may be carried out on preparations containing mRNA or cDNA generated from isolated mRNA in a manner that reflects the relative levels of mRNA transcripts in the sample. Levels of RNA can be determined specific amplification reaction such as PCR using one or more pairs of primers may be employed to amplify a region of the nucleic acid, and preferably a region with less homology to other genes. Nucleic acid for testing may be prepared from nucleic acid removed from cells or in a library using a variety of other techniques such as restriction enzyme digest and electrophoresis.

Nucleic acid may be screened using a TTK-specific probe. Such a probe corresponds in sequence to a region of the TTK gene, or its complement. Under stringent conditions, specific hybridization of such a probe to test nucleic acid is indicative of the presence of the TTK nucleic acid in a sample. For efficient screening purposes, more than one probe may be used on the same test sample. The probe may contain as few as 15, 20, 50 or 100 nucleotides of the TTK gene of SEQ ID. No. 13 or may be as long as or 500, 1 kb or as much as 3.8 kb or longer in length.

Allele- or variant-specific oligonucleotides may similarly be used in PCR to specifically amplify particular sequences if present in a test sample. Assessment of whether a PCR band contains a gene variant may be carried out in a number of ways familiar to those skilled in the art. The PCR product may for instance be treated in a way that enables one to display the mutation or polymorphism on a denaturing polyacrylamide DNA sequencing gel, with specific bands that are linked to the gene variants being selected. This can be done simultaneous to or sequentially to determining the level of a normal TTK sequence, *e.g.*, to determine the combinatory levels of total TTK.

The presence of absence of a lesion in a promoter or other regulatory sequence may also be assessed by determining the level of mRNA production by transcription or the level of polypeptide production by translation from the mRNA. The presence of differences in sequence of nucleic acid molecules may be detected by means of restriction enzyme digestion, such as in a method of DNA fingerprinting where the restriction pattern produced when one or more restriction enzymes are used to cut a sample of nucleic acid is compared with the pattern

obtained when a sample containing the normal gene or a variant or allele is digested with the same enzyme or enzymes.

A test sample of nucleic acid may be provided for example by extracting nucleic acid from cells, e.g., cells from a tumor biopsy.

#### Detection of TTK polypeptides

There are various methods for determining the presence or absence in a test sample of a TTK polypeptide. A sample may be tested for the presence of a binding partner for a specific binding member such as an antibody (or mixture of antibodies), specific for wild-type TTK and/or one or more particular variants (e.g., allelic variants) of the TTK polypeptide. In such cases, the sample may be tested by being contacted with a specific binding member such as an antibody under appropriate conditions for specific binding. Where a panel of antibodies is used, different reporting labels may be employed for each antibody so that binding of each can be determined. In addition to detection of TTK polypeptides using anti-TTK antibodies, TTK polypeptide can also be identified using TTK-specific activity assays.

#### Arrays

Binding agents (such as antibodies or nucleic acid sequences) can also be immobilized in small, discrete locations and/or as arrays on solid supports or on diagnostic chips. These approaches can be particularly valuable as they can provide great sensitivity, particularly through the use of fluorescently labeled reagents, require only very small amounts of biological sample from individuals being tested and allow a variety of separate assays can be carried out simultaneously. This latter advantage can be useful as it provides an assay for different proteins (e.g., an oncogene or tumor suppressor) in tandem with the assay for TTK. Thus, in a further aspect, the present invention provides a support or diagnostic chip having immobilized thereon one or more binding agents capable of specifically binding TTK nucleic acid or polypeptides, optionally in combination with other reagents needed to carrying out an assay.

#### Methods for expression of TTK polypeptide

The full-length or partial polypeptides encoded by TTK may be expressed in any expression system, including, for example, bacterial, yeast, insect, amphibian and mammalian systems. Suitable vectors and host cells for which are described in U.S. Patent No. 5,654,173. Appropriate polynucleotide constructs are purified using standard recombinant DNA techniques as described in, for example, Sambrook *et al.*, (1989) *Molecular Cloning: A*

*Laboratory Manual*, 2nd ed. (Cold Spring Harbor Press, Cold Spring Harbor, New York), and under current regulations described in United States Dept. of HHS, National Institute of Health (NIH) Guidelines for Recombinant DNA Research.

Bacteria. Expression systems in bacteria include those described in Chang *et al.*, *Nature* (1978) 275:615, Goeddel *et al.*, *Nature* (1979) 281:544, Goeddel *et al.*, *Nucleic Acids Res.* (1980) 8:4057; EP 0 036,776, U.S. Patent No. 4,551,433, DeBoer *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1983) 80:21-25, and Siebenlist *et al.*, *Cell* (1980) 20:269.

Yeast. Expression systems in yeast include those described in Hinnen *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1978) 75:1929; Ito *et al.*, *J. Bacteriol.* (1983) 153:163; Kurtz *et al.*, *Mol. Cell. Biol.* (1986) 6:142; Kunze *et al.*, *J. Basic Microbiol.* (1985) 25:141; Gleeson *et al.*, *J. Gen. Microbiol.* (1986) 132:3459, Roggenkamp *et al.*, *Mol. Gen. Genet.* (1986) 202:302) Das *et al.*, *J. Bacteriol.* (1984) 158:1165; De Louvencourt *et al.*, *J. Bacteriol.* (1983) 154:737, Van den Berg *et al.*, *Bio/Technology* (1990) 8:135; Kunze *et al.*, *J. Basic Microbiol.* (1985) 25:141; Cregg *et al.*, *Mol. Cell. Biol.* (1985) 5:3376, U.S. Patent Nos. 4,837,148 and 4,929,555; Beach and Nurse, *Nature* (1981) 300:706; Davidow *et al.*, *Curr. Genet.* (1985) 10:380, Gaillardin *et al.*, *Curr. Genet.* (1985) 10:49, Ballance *et al.*, *Biochem. Biophys. Res. Commun.* (1983) 112:284-289; Tilburn *et al.*, *Gene* (1983) 26:205-221, Yelton *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1984) 81:1470-1474, Kelly and Hynes, *EMBO J.* (1985) 4:475479; EP 0 244,234, and WO 91/00357.

Insect Cells. Expression of heterologous genes in insects is accomplished as described in U.S. Patent No. 4,745,051, Friesen *et al.* (1986) "The Regulation of Baculovirus Gene Expression" in: *The Molecular Biology Of Baculoviruses* (W. Doerfler, ed.), EP 0 127,839, EP 0 155,476, and Vlak *et al.*, *J. Gen. Virol.* (1988) 69:765-776, Miller *et al.*, *Ann. Rev. Microbiol.* (1988) 42:177, Carbonell *et al.*, *Gene* (1988) 73:409, Maeda *et al.*, *Nature* (1985) 315:592-594, Lebacq-Verheyden *et al.*, *Mol. Cell. Biol.* (1988) 8:3129; Smith *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1985) 82:8404, Miyajima *et al.*, *Gene* (1987) 58:273; and Martin *et al.*, *DNA* (1988) 7:99. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts are described in Luckow *et al.*, *Bio/Technology* (1988) 6:47-55, Miller *et al.*, *Generic Engineering* (Setlow, J.K. *et al.* eds.), Vol. 8 (Plenum Publishing, 1986), pp. 277-279, and Maeda *et al.*, *Nature*, (1985) 315:592-594.

Mammalian Cells. Mammalian expression is accomplished as described in Dijkema *et al.*, *EMBO J.* (1985) 4:761, Gorman *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1982) 79:6777,



Boshart *et al.*, *Cell* (1985) 41:521 and U.S. Patent No. 4,399,216. Other features of mammalian expression are facilitated as described in Ham and Wallace, *Meth. Enz.* (1979) 58:44, Barnes and Sato, *Anal. Biochem.* (1980) 102:255, U.S. Patent Nos. 4,767,704, 4,657,866, 4,927,762, 4,560,655, WO 90/103430, WO 87/00195, and U.S. RE 30,985.

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#### Screening Assays to Identify Chemotherapeutic Agents

The invention also encompasses screening assays to identify agents that modulate TTK activity, specifically that decrease aberrant TTK activity in an affected cell, *e.g.*, a cancerous or pre-cancerous cell in which TTK is differentially expressed. Such assays may be performed either *in vitro* or *in vivo*.

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#### Candidate Agents

The term "agent" as used herein describes any molecule with the capability of altering the expression or physiological function of a gene product of a differentially expressed gene. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, *i.e.*, at zero concentration or below the level of detection.

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Candidate agents encompass numerous chemical classes, including, but not limited to, organic molecules (*e.g.*, small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons), peptides, monoclonal antibodies, antisense polynucleotides, and ribozymes, and the like. Candidate agents can comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including, but not limited to: polynucleotides, peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

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Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of

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natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs. Candidate agents can be assessed for modulation of TTK activity either singly or in pools.

#### Screening of Candidate Agents *In Vitro*

A wide variety of *in vitro* assays may be used to screen candidate agents for the desired biological activity, including, but not limited to, labeled *in vitro* protein-protein binding assays, protein-DNA binding assays (*e.g.*, to identify agents that affect expression), electrophoretic mobility shift assays, immunoassays for protein binding, and the like. For example, by providing for the production of large amounts of a differentially expressed polypeptide, one can identify ligands or substrates that bind to, modulate or mimic the action of the polypeptide. Further methods for identifying these ligands and substrates are provided below. The purified polypeptide may also be used for determination of three-dimensional crystal structure, which can be used for modeling intermolecular interactions, transcriptional regulation, etc.

The screening assay can be a binding assay, wherein one or more of the molecules may be joined to a label, and the label directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles, *e.g.*, magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

A variety of other reagents may be included in the screening assays described herein. Where the assay is a binding assay, these include reagents like salts, neutral proteins, *e.g.*, albumin, detergents, etc that are used to facilitate optimal protein-protein binding, protein-DNA binding, and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc. may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to

facilitate rapid high-throughput screening. Typically between 0.1 and 1 hours will be sufficient.

Many mammalian genes have homologs in yeast and lower animals. The study of such homologs physiological role and interactions with other proteins *in vivo* or *in vitro* can facilitate understanding of biological function. In addition to model systems based on genetic complementation, yeast has been shown to be a powerful tool for studying protein-protein interactions through the two hybrid system described in Chien et al. 1991 *Proc. Natl. Acad. Sci. USA* 88:9578-9582.

#### Screening of Candidate Agents *In Vivo*

Candidate agents can be screened in a non-human animal model of cancer (*e.g.*, in animals into which have been injected cancerous cells; in animals that are transgenic for an alteration in expression of a differentially expressed gene as described herein, *e.g.*, a transgenic "knock-out," or a transgenic "knock-in," a polynucleotide encoding all or a portion of a differentially expressed gene product and comprising an operably linked reporter gene, and the like).

In general, the candidate agent is administered to the animal, and the effects of the candidate agent determined. The candidate agent can be administered in any manner desired and/or appropriate for delivery of the agent in order to effect a desired result. For example, the candidate agent can be administered by injection (*e.g.*, by injection intravenously, intramuscularly, subcutaneously, or directly into the tissue in which the desired affect is to be achieved), orally, or by any other desirable means. Normally, the *in vivo* screen will involve a number of animals receiving varying amounts and concentrations of the candidate agent (from no agent to an amount of agent hat approaches an upper limit of the amount that can be delivered successfully to the animal), and may include delivery of the agent in different formulation. The agents can be administered singly or can be combined in combinations of two or more, especially where administration of a combination of agents may result in a synergistic effect.

The effect of agent administration upon the transgenic animal can be monitored by assessing expression of the gene product, growth of the injected tumor cells, and the like.

#### Identified Candidate Agents

Compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host for treatment of a condition that is amenable to

treatment by modulation of expression of a differentially expressed gene product. The therapeutic agents may be administered in a variety of ways, orally, topically, parenterally *e.g.*, subcutaneously, intraperitoneally, by viral infection, intravascularly, etc. Oral and inhaled treatments are of particular interest. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%. The therapeutic agents can be administered in a single dose, or as multiple doses over a course of treatment.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents.

#### Methods of Screening for Drugs that Modulate TTK Activity

A TTK polypeptide or TTK-encoding nucleic acid according to the present invention may be used in screening for molecules which affect or modulate TTK activity or function. Such molecules may be useful in a therapeutic and/or prophylactic context. Means for screening for substances potentially useful in treating or preventing cancer is provided by the present invention. In general, the methods of the invention are to facilitate identification of modulators of TTK activity (*e.g.*, by modulating activity of TTK polypeptide or other TTK gene product, or by affecting TTK activity by targeting activity of gene products that act either upstream or downstream of TTK in a cascade that leads to TTK activity), with agents that decrease TTK activity generally being of particular interest. Substances identified as modulators of the TTK activity represent an advance in the fight against cancer since they provide basis for design and investigation of pharmaceuticals for *in vivo* use.

A method of screening for a substance which modulates activity of a polypeptide may include contacting one or more test substances with the polypeptide in a suitable reaction medium, testing the activity of the treated polypeptide (*e.g.*, the ability to phosphorylate its substrate) and comparing that activity with the activity of the polypeptide in comparable reaction medium untreated with the test substance or substances. A difference in activity

between the treated and untreated polypeptides is indicative of a modulating effect of the relevant test substance or substances.

Combinatorial library technology provides an efficient way of testing a potentially vast number of different substances for ability to modulate activity of a polypeptide. Such libraries and their use are known in the art. The use of peptide libraries is preferred. Test substances may also be screened for ability to interact with the polypeptide, *e.g.*, in a yeast two-hybrid system. This may be used as a coarse screen prior to testing a substance for actual ability to modulate activity of the polypeptide. Alternatively, the screen could be used to screen test substances for binding to a TTK specific binding partner.

A substance identified using as a modulator of TTK polypeptide function may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many *in vivo* pharmaceutical uses. Accordingly, a mimetic or mimic of the substance (particularly if a peptide) may be designed for pharmaceutical use.

#### TTK activity assays

The activity of the TTK may be measured using any suitable kinase assay known in the art. For example, and not by way of limitation, the methods described in Hogg et al (Oncogene 1994 9:98-96), Mills et al (J. Biol. Chem. 1992 267:16000-006) and Tomizawa et al 2001 (FEBS Lett. 2001 492: 221-7), Schmandt et al, (J. Immunol. 1994, 152:96-105) may be used. Further serine, threonine and tyrosine kinase assays are described in Ausubel et al. (Short Protocols in Molecular Biology, 1999, unit 17.6).

TTK assays generally use TTK polypeptide, a labeled donor substrate, and a receptor substrate that is either specific or non-specific for TTK. In such assays TTK transfers a labeled moiety from the donor substrate to the receptor substrate, and kinase activity is measured by the amount of labeled moiety transferred from the donor substrate to the receptor substrate.

TTK polypeptide may be produced using various expression systems as detailed above, may be purified from cells, may be in the form of a cleaved or uncleaved recombinant fusion protein and may have non-TTK polypeptide sequences, for example a His tag or  $\beta$ -galactosidase at its N- or C-terminus. TTK activity may be assayed in cancerous cells lines if the cancerous cell lines are used as a source of the TTK to be assayed. Suitable donor substrates for TTK assays include any molecule that is susceptible to dephosphorylation by TTK include  $\gamma$ -labeled ATP and ATP analogs, wherein the label is  $^{33}\text{P}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$  or any other radioactive isotope or a suitable fluorescent marker. Suitable recipient substrates for TTK

assays include any polypeptide or other molecule that is susceptible to phosphorylation by TTK. Recipient substrates are usually derived from fragments of *in vivo* targets of TTK. Recipient substrates fragments may be 8 to 50 amino acids in length, usually 10 to 30 amino acids and preferably of about 10, 12, 15, 18, 20 and 25 amino acids in length. Further recipient substrates can be determined empirically using a set of different polypeptides or other molecules. Targets of TTK suitable for TTK assays include tau and cdc25. Recipient substrates for TTK are typically capable of being purified from other components of the reaction once the reaction has been performed. This purification is usually done through a molecular interaction, where the recipient substrates is biotinylated and purified through its interaction with streptavidin, or a specific antibody is available that can specifically recognize the recipient substrates. The reaction can be performed in a variety of conditions, such as on a solid support, in a gel, in solution or in living cells.

One exemplary recipient substrate for TTK phosphorylation is the human protein cdc25, SEQ ID NO:26, which is phosphorylated by TTK at the serine residues of amino acid position 214 and 216. Two fragments of cdc25 are used as substrates in the kinase assay described below. These fragments comprise peptides A (SEQ ID NO:27), corresponding to amino acids 209 to 225 of the cdc25 polypeptide sequence or peptide B (SEQ ID NO:28), corresponds to amino acids 210 to 223 of the cdc25 polypeptide. In this assay, two biotinylated polypeptides of comprising either SEQ ID NO:27 (Biotin-SGSGSGLYRSPSPENLNRPR-NH<sub>2</sub>) or SEQ ID NO:28 (Biotin-GGGGLYRSPSPENLNRK-OH) are used.

The choice of detection methods depends on type of label used for the donor molecule and may include, for example, measurement of incorporated radiation or fluorescence by autoradiography, scintillation, scanning or fluorography.

#### Methods of Inhibiting Tumor Growth and Other Treatment Goals

The invention further provides methods for reducing growth of cancer cells, particular breast or colon cancer cells. In general, the methods comprise contacting a cancer cell that expresses TTK at an aberrant level relative to normal cells with a substance that (1) modulates, generally decreases, expression of TTK (*e.g.*, a antisense polynucleotide corresponding to TTK); or (2) otherwise modulates, generally decreases, TTK polypeptide levels and/or TTK activity in a cancerous cell having aberrant TTK activity.

“Reducing growth of a cancer cell” includes, but is not limited to, reducing proliferation of cancer cells, and reducing the incidence of a normal cell from developing a cancerous phenotype or morphology. Whether a reduction in cancer cell growth has been achieved can be readily determined using any known assay, including, but not limited to, [<sup>3</sup>H]-thymidine incorporation; counting cell number over a period of time; detecting, measuring a marker associated with colon cancer (e.g., CEA, CA19-9, and LASA), and/or methods well known in the art for assessing tumor burden.

The present invention provides methods for treating cancer (particularly breast and colon cancer or other cancer that is associated with aberrantly high TTK activity) which methods generally comprise administering to an individual an agent that reduces TTK activity in an amount sufficient to reduce cancer cell growth to treat the cancer. Whether a substance, or a specific amount of the substance, is effective in treating cancer can be assessed using any of a variety of known diagnostic assays, *e.g.*, in the case of colon cancer, sigmoidoscopy, proctoscopy, rectal examination, colonoscopy with biopsy, contrast radiographic studies, CAT scans, angiography, and detection of a tumor marker associated with colon cancer in the blood of the individual. The substance can be administered systemically or locally. Thus, in some embodiments, the substance is administered locally, and colon cancer growth is decreased at the site of administration. Local administration may be useful in treating, *e.g.*, a solid tumor.

In one embodiment, the invention features polynucleotides that act as antisense polynucleotides and decrease TTK activity. Antisense TTK polynucleotides generally comprise a polynucleotide of at least about 20 to 3000 nucleotides, usually at least about 20 to 1000 nucleotides and more usually at least about 8 to 50 nucleotides, and preferably about 26, 20, 18, 17, 15, 10 and 8 nucleotides. Exemplary TTK polynucleotides are provided in the Examples and in SEQ ID NO:1-12, although any antisense fragment of SEQ ID NO:13 will suffice. .

The therapeutic regimen is selected according to the expression profile. For example, if a patient's tumor indicates that the tumor produces aberrantly high level of TTK relative to normal cells, then a drug having efficacy in the treatment of such TTK-expressing tumors is selected for therapy of that patient.

Pharmaceutical compositions

Pharmaceutical compositions of the invention can comprise a therapeutically effective amount of a polypeptide, antibody, polynucleotide (including antisense nucleotides and ribozymes), or small molecule or other compound identified as modulating activity of TTK, preferably decreasing TTK activity. The term “therapeutically effective amount” as used herein refers to an amount of a therapeutic agent to treat, ameliorate, or prevent a desired disease or condition, or to exhibit a detectable therapeutic or preventative effect. The effect can be detected by, for example, chemical markers or antigen levels. Therapeutic effects also include reduction in physical symptoms, such as decreased body temperature, and/or in the effect upon tumor load in the subject (*e.g.*, decrease in tumor size or inhibition in tumor growth). The precise effective amount for a subject will depend upon the subject's size and health, the nature and extent of the condition, and the therapeutics or combination of therapeutics selected for administration. Thus, it is not useful to specify an exact effective amount in advance. However, the effective amount for a given situation is determined by routine experimentation and is within the judgment of the clinician. For purposes of the present invention, an effective dose will generally be from about 0.01 mg/ kg to 50 mg/kg or 0.05 mg/kg to about 10 mg/kg of the DNA constructs in the individual to which it is administered.

A pharmaceutical composition can also contain a pharmaceutically acceptable carrier. The term “pharmaceutically acceptable carrier” refers to a carrier for administration of a therapeutic agent, such as antibodies or a polypeptide, genes, and other therapeutic agents. The term refers to any pharmaceutical carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which can be administered without undue toxicity. Suitable carriers can be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Pharmaceutically acceptable carriers in therapeutic compositions can include liquids such as water, saline, glycerol and ethanol. Auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, can also be present in such vehicles. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. Liposomes are included within the definition



of a pharmaceutically acceptable carrier. Pharmaceutically acceptable salts can also be present in the pharmaceutical composition, e.g., mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in *Remington's Pharmaceutical Sciences* (Mack Pub. Co., N.J. 1991). The precise nature of the carrier or other material may depend on the route of administration, e.g., oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is has suitable pH, isotonicity and stability. Suitable solutions, for example, optionally include but are not limited to isotonic vehicles such as sodium chloride, preservatives, stabilizers, buffers, antioxidants and/or other additives as required.

Administration of the pharmaceutical is administered in a prophylactically effective amount or a therapeutically effective amount. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Decisions on dosage etc, can be determined by one skilled in the art based upon the disclosed methods, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in *Remington's Pharmaceutical Sciences*, 16th edition, Osol, A. (ed), 1980.

Alternatively, targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons; for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells. Targeting can be accomplished by, for

example, administering a drug-antibody complex to a subject, wherein the antibody is specific for a cancer-associated antigen, and the drug is one that reduces cancer cell growth. Targeting can be accomplished by coupling (e.g., linking, directly or via a linker molecule, either covalently or non-covalently, so as to form a drug-antibody complex) a drug to an antibody specific for a cancer-associated antigen. Methods of coupling a drug to an antibody are well known in the art and need not be elaborated upon herein.

Pharmaceutical agents can also be produced in the target cells by expression from an encoding gene introduced into the cells, *e.g.*, in a viral or liposomal vector. The vector could be targeted to the specific cells to be treated, or it could contain regulatory elements which are switched on more or less selectively by the target cells.

Alternatively, the agent could be administered in a precursor form, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

#### Delivery Methods for Therapy

Once formulated, the compositions of the invention or identified using the methods of the invention can be administered directly to the subject (*e.g.*, as polynucleotide or polypeptides). Direct delivery of the compositions will generally be accomplished by parenteral injection, *e.g.*, subcutaneously, intraperitoneally, intravenously or intramuscularly, intratumoral or to the interstitial space of a tissue. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal applications, needles, and gene guns or hyposprays. Dosage treatment can be a single dose schedule or a multiple dose schedule.

Once a gene corresponding to a polynucleotide of the invention has been found to correlate with a proliferative disorder, such as neoplasia, dysplasia, and hyperplasia, the disorder can be amenable to treatment by administration of a therapeutic agent based on the provided polynucleotide, corresponding polypeptide or other corresponding molecule (*e.g.*, antisense, ribozyme, etc.).

The dose and the means of administration are determined based on the specific qualities of the therapeutic composition, the condition, age, and weight of the patient, the progression of the disease, and other relevant factors. For example, administration of polynucleotide

therapeutic compositions agents of the invention includes local or systemic administration, including injection, oral administration, particle gun or catheterized administration, and topical administration. Preferably, the therapeutic polynucleotide composition contains an expression construct comprising a promoter operably linked to a polynucleotide of at least 12, 15, 17, 18, 22, 25, 30, or 35 contiguous -nucleotides of the polynucleotide disclosed herein. Various methods can be used to administer the therapeutic composition directly to a specific site in the body. For example, a small metastatic lesion is located and the therapeutic composition injected several times in several different locations within the body of tumor. Alternatively, arteries which serve a tumor are identified, and the therapeutic composition injected into such an artery, in order to deliver the composition directly into the tumor. A tumor that has a necrotic center is aspirated and the composition injected directly into the now empty center of the tumor. The antisense composition is directly administered to the surface of the tumor, for example, by topical application of the composition. X-ray imaging is used to assist in certain of the above delivery methods.

Receptor-mediated targeted delivery of therapeutic compositions containing an antisense polynucleotide, subgenomic polynucleotides, or antibodies to specific tissues can also be used. Receptor-mediated DNA delivery techniques are described in, for example, Findeis *et al.*, *Trends Biotechnol.* (1993) 11:202; Chiou *et al.*, *Gene Therapeutics: Methods And Applications Of Direct Gene Transfer* (J.A. Wolff, ed.) (1994); Wu *et al.*, *J. Biol. Chem.* (1988) 263:621; Wu *et al.*, *J. Biol. Chem.* (1994) 269:542; Zenke *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1990) 87:3655; Wu *et al.*, *J. Biol. Chem.* (1991) 266:338. Therapeutic compositions containing a polynucleotide are administered in a range of about 100 ng to about 200 mg of DNA for local administration in a gene therapy protocol. Concentration ranges of about 500 ng to about 50 mg, about 1  $\mu$ g to about 2 mg, about 5  $\mu$ g to about 500  $\mu$ g, and about 20  $\mu$ g to about 100  $\mu$ g of DNA can also be used during a gene therapy protocol. Factors such as method of action (e.g., for enhancing or inhibiting levels of the encoded gene product) and efficacy of transformation and expression are considerations which will affect the dosage required for ultimate efficacy of the antisense subgenomic polynucleotides. Where greater expression is desired over a larger area of tissue, larger amounts of antisense subgenomic polynucleotides or the same amounts readministered in a successive protocol of administrations, or several administrations to different adjacent or close tissue portions of, for example, a tumor site, may be required to effect a positive therapeutic outcome. In all cases,

routine experimentation in clinical trials will determine specific ranges for optimal therapeutic effect. For polynucleotide related genes encoding polypeptides or proteins with anti-inflammatory activity, suitable use, doses, and administration are described in USPN 5,654,173.

5       The therapeutic polynucleotides and polypeptides of the present invention can be delivered using gene delivery vehicles. The gene delivery vehicle can be of viral or non-viral origin (see generally, Jolly, *Cancer Gene Therapy* (1994) 1:51; Kimura, *Human Gene Therapy* (1994) 5:845; Connelly, *Human Gene Therapy* (1995) 1:185; and Kaplitt, *Nature Genetics* (1994) 6:148). Expression of such coding sequences can be induced using endogenous  
10       mammalian or heterologous promoters. Expression of the coding sequence can be either constitutive or regulated.

      Viral-based vectors for delivery of a desired polynucleotide and expression in a desired cell are well known in the art. Exemplary viral-based vehicles include, but are not limited to, recombinant retroviruses (see, e.g., WO 90/07936; WO 94/03622; WO 93/25698; WO  
15       93/25234; USPN 5, 219,740; WO 93/11230; WO 93/10218; USPN 4,777,127; GB Patent No. 2,200,651; EP 0 345 242; and WO 91/02805), alphavirus-based vectors (e.g., Sindbis virus vectors, Semliki forest virus (ATCC VR-67; ATCC VR-1247), Ross River virus (ATCC VR-373; ATCC VR-1246) and Venezuelan equine encephalitis virus (ATCC VR-923; ATCC VR-1250; ATCC VR 1249; ATCC VR-532), and adeno-associated virus (AAV) vectors (see, e.g.,  
20       WO 94/12649, WO 93/03769; WO 93/19191; WO 94/28938; WO 95/11984 and WO 95/00655). Administration of DNA linked to killed adenovirus as described in Curiel, *Hum. Gene Ther.* (1992) 3:147 can also be employed.

      Non-viral delivery vehicles and methods can also be employed, including, but not limited to, polycationic condensed DNA linked or unlinked to killed adenovirus alone (see,  
25       e.g., Curiel, *Hum. Gene Ther.* (1992) 3:147); ligand-linked DNA (see, e.g., Wu, *J. Biol. Chem.* (1989) 264:16985); eukaryotic cell delivery vehicles (see, e.g., USPN 5,814,482; WO 95/07994; WO 96/17072; WO 95/30763; and WO 97/42338) and nucleic acid neutralization or fusion with cell membranes. Naked DNA can also be employed. Exemplary naked DNA introduction methods are described in WO 90/11092 and USPN 5,580,859.  
30       Liposomes that can act as gene delivery vehicles are described in USPN 5,422,120; WO 95/13796; WO 94/23697; WO 91/14445; and EP 0524968. Additional approaches are described in Philip, *Mol. Cell Biol.* (1994) 14:2411, and in Woffendin, *Proc. Natl. Acad. Sci.*

(1994) 91:1581.

Further non-viral delivery suitable for use includes mechanical delivery systems such as the approach described in Woffendin *et al.*, *Proc. Natl. Acad. Sci. USA* (1994) 91(24):11581. Moreover, the coding sequence and the product of expression of such can be delivered through deposition of photopolymerized hydrogel materials or use of ionizing radiation (see, e.g., USPN 5,206,152 and WO 92/11033). Other conventional methods for gene delivery that can be used for delivery of the coding sequence include, for example, use of hand-held gene transfer particle gun (see, e.g., USPN 5,149,655); use of ionizing radiation for activating transferred gene (see, e.g., USPN 5,206,152 and WO 92/11033).

As an alternative to the use of viral vectors other known methods of introducing nucleic acid into cells includes electroporation, calcium phosphate co-precipitation, mechanical techniques such as microinjection, transfer mediated by liposomes and direct DNA uptake and receptor-mediated DNA transfer. Gene transfer techniques which selectively target the TTK nucleic acid to the affected cell type are preferred. Examples of this included receptor-mediated gene transfer, in which the nucleic acid is linked to a protein ligand via polylysine, with the ligand being specific for a receptor present on the surface of the target cells.

#### Screening for Substances Affecting TTK Expression

The present invention also provides the use of all or part of the nucleic acid sequence of the TTK promoter and/or enhancer regions in methods of screening for substances which modulate the activity of the promoter and increase or decrease the level of TTK expression. This assay can be performed to identify anti-cancer agents for therapeutic and/or prophylactic purposes. The level of promoter activity, *i.e.*, the ability to initiate transcription, is quantifiable for instance by assessment of the amount of mRNA produced by transcription from the promoter or by assessment of the amount of protein product produced by translation of mRNA produced by transcription from the promoter. The amount of a specific mRNA present in an expression system may be determined for example using specific oligonucleotides which are able to hybridize with the mRNA and which are labeled or may be used in a specific amplification reaction such as PCR. Use of a reporter gene facilitates determination of promoter activity by reference to protein production.

Generally, a reporter gene under control of the TTK promoter and/or enhancers may be transcribed into mRNA which may be translated into a peptide or polypeptide product which

may be detected and preferably quantitated following expression. The reporter gene preferably encodes an enzyme which catalyses a reaction which produces a detectable signal, preferably a visually detectable signal, such as a coloured product. Many examples are known, including  $\beta$ -galactosidase and luciferase.  $\beta$ -galactosidase activity may be assayed by production of blue color on substrate, the assay being by eye or by use of a spectrophotometer to measure absorbance. Fluorescence, for example that produced as a result of luciferase activity, may be quantitated using a spectrophotometer. Radioactive assays may be used, for instance using choloramphenicol acetyltransferase, which may also be used in non-radioactive assays. The presence and/or amount of gene product resulting from expression from the reporter gene may be determined using a molecule able to bind the product, such as an antibody or fragment thereof. The binding molecule may be labeled directly or indirectly using any standard technique.

Those skilled in the art are well aware of a multitude of possible reporter genes and assay techniques which may be used to determine gene activity according to the presently disclosed methods. Any suitable reporter/assay may be used and the present invention is intended to encompass such systems.

Following identification of a substance which modulates or affects promoter activity, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, *i.e.* manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug.

#### Integrated Disease Information System

The levels of TTK in a sample can be used in an integrated disease information system to aid in analysis such as proposed patient interventions, designing clinical trials, performing pharmacoeconomic analysis, and illustrating disease progression for various patients over time. For example, TTK information determined according to the methods of the invention can be used in a system such as that described in U.S. Pat. No. 6,108,635 issued to Herren, et al. on August 22, 2000. Such a system can be for collecting the results of medical treatments given to patients in a plurality of locations. *See, e.g.,* U.S. Pat. No. 5,713,350 issued to Yokota, et al. on February 3, 1998.

## EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

### EXAMPLE 1: SOURCE OF PATIENT TISSUE SAMPLES

Normal and cancerous tissues were collected from patients using laser capture microdissection (LCM) techniques, which techniques are well known in the art (see, *e.g.*, Ohyama *et al.* (2000) *Biotechniques* 29:530-6; Curran *et al.* (2000) *Mol. Pathol.* 53:64-8; Suarez-Quian *et al.* (1999) *Biotechniques* 26:328-35; Simone *et al.* (1998) *Trends Genet* 14:272-6; Conia *et al.* (1997) *J. Clin. Lab. Anal.* 11:28-38; Emmert-Buck *et al.* (1996) *Science* 274:998-1001). Table 1 (inserted following the last page of the Examples ) provides information about each patient from which the samples were isolated, including: the Patient ID and Path ReportID, numbers assigned to the patient and the pathology reports for identification purposes; the anatomical location of the tumor (AnatomicalLoc); The Primary Tumor Size; the Primary Tumor Grade; the Histopathologic Grade; a description of local sites to which the tumor had invaded (Local Invasion); the presence of lymph node metastases (Lymph Node Metastasis); incidence of lymph node metastases (provided as number of lymph nodes positive for metastasis over the number of lymph nodes examined) (Incidence Lymphnode Metastasis); the Regional Lymphnode Grade; the identification or detection of metastases to sites distant to the tumor and their location (Distant Met & Loc); a description of the distant metastases (Description Distant Met); the grade of distant metastasis (Distant Met Grade); and general comments about the patient or the tumor (Comments). Adenoma was not described in any of the patients; adenoma dysplasia (described as hyperplasia by the pathologist) was described in Patient ID No. 695. Extranodal extensions were described in two patients, Patient ID Nos. 784 and 791. Lymphovascular invasion was described in seven

patients, Patient ID Nos. 128, 278, 517, 534, 784, 786, and 791.. Crohn's-like infiltrates were described in seven patients, Patient ID Nos. 52, 264, 268, 392, 393, 784, and 791.

#### EXAMPLE 2: DIFFERENTIAL EXPRESSION OF TTK

5 cDNA probes were prepared from total RNA isolated from the patient cells described in Example 1. Since LCM provides for the isolation of specific cell types to provide a substantially homogenous cell sample, this provided for a similarly pure RNA sample.

Total RNA was first reverse transcribed into cDNA using a primer containing a T7 RNA polymerase promoter, followed by second strand DNA synthesis. cDNA was then  
 10 transcribed *in vitro* to produce antisense RNA using the T7 promoter-mediated expression (see, e.g., Luo *et al.* (1999) *Nature Med* 5:117-122), and the antisense RNA was then converted into cDNA. The second set of cDNAs were again transcribed *in vitro*, using the T7 promoter, to provide antisense RNA. Optionally, the RNA was again converted into cDNA, allowing for up to a third round of T7-mediated amplification to produce more antisense RNA. Thus the  
 15 procedure provided for two or three rounds of *in vitro* transcription to produce the final RNA used for fluorescent labeling. Fluorescent probes were generated by first adding control RNA to the antisense RNA mix, and producing fluorescently labeled cDNA from the RNA starting material. Fluorescently labeled cDNAs prepared from the tumor RNA sample were compared to fluorescently labeled cDNAs prepared from normal cell RNA sample. For example, the  
 20 cDNA probes from the normal cells were labeled with Cy3 fluorescent dye (green) and the cDNA probes prepared from the tumor cells were labeled with Cy5 fluorescent dye (red).

Each array used had an identical spatial layout and control spot set. Each microarray was divided into two areas, each area having an array with, on each half, twelve groupings of 32 x 12 spots for a total of about 9,216 spots on each array. The two areas are spotted  
 25 identically which provide for at least two duplicates of each clone per array. Spotting was accomplished using PCR amplified products from 0.5kb to 2.0 kb and spotted using a Molecular Dynamics Gen III spotter according to the manufacturer's recommendations. The first row of each of the 24 regions on the array had about 32 control spots, including 4 negative control spots and 8 test polynucleotides. The test polynucleotides were spiked into each  
 30 sample before the labeling reaction with a range of concentrations from 2-600 pg/slide and ratios of 1:1 . For each array design, two slides were hybridized with the test samples reverse-



labeled in the labeling reaction. This provided for about 4 duplicate measurements for each clone, two of one color and two of the other, for each sample.

The differential expression assay was performed by mixing equal amounts of probes from tumor cells and normal cells of the same patient. The arrays were prehybridized by incubation for about 2 hrs at 60°C in 5X SSC/0.2% SDS/1 mM EDTA, and then washed three times in water and twice in isopropanol. Following prehybridization of the array, the probe mixture was then hybridized to the array under conditions of high stringency (overnight at 42°C in 50% formamide, 5X SSC, and 0.2% SDS. After hybridization, the array was washed at 55°C three times as follows: 1) first wash in 1X SSC/0.2% SDS; 2) second wash in 0.1X SSC/0.2% SDS; and 3) third wash in 0.1X SSC.

The arrays were then scanned for green and red fluorescence using a Molecular Dynamics Generation III dual color laser-scanner/detector. The images were processed using BioDiscovery Autogene software, and the data from each scan set normalized to provide for a ratio of expression relative to normal. Data from the microarray experiments was analyzed according to the algorithms described in U.S. application serial no. 60/252,358, filed November 20, 2000, by E.J. Moler, M.A. Boyle, and F.M. Randazzo, and entitled "Precision and accuracy in cDNA microarray data," which application is specifically incorporated herein by reference.

The experiment was repeated, this time labeling the two probes with the opposite color in order to perform the assay in both "color directions." Each experiment was sometimes repeated with two more slides (one in each color direction). The level fluorescence for each sequence on the array expressed as a ratio of the geometric mean of 8 replicate spots/genes from the four arrays or 4 replicate spots/gene from 2 arrays or some other permutation. The data were normalized using the spiked positive controls present in each duplicated area, and the precision of this normalization was included in the final determination of the significance of each differential. The fluorescent intensity of each spot was also compared to the negative controls in each duplicated area to determine which spots have detected significant expression levels in each sample.

A statistical analysis of the fluorescent intensities was applied to each set of duplicate spots to assess the precision and significance of each differential measurement, resulting in a p-value testing the null hypothesis that there is no differential in the expression level between the tumor and normal samples of each patient. During initial analysis of the microarrays, the

hypothesis was accepted if  $p > 10^{-3}$ , and the differential ratio was set to 1.000 for those spots. All other spots have a significant difference in expression between the tumor and normal sample. If the tumor sample has detectable expression and the normal does not, the ratio is truncated at 1000 since the value for expression in the normal sample would be zero, and the ratio would not be a mathematically useful value (e.g., infinity). If the normal sample has detectable expression and the tumor does not, the ratio is truncated to 0.001, since the value for expression in the tumor sample would be zero and the ratio would not be a mathematically useful value. These latter two situations are referred to herein as "on/off." Database tables were populated using a 95% confidence level ( $p > 0.05$ ).

The difference in the expression level of TTK in the colon tumor cells relative to the matched normal colon cells was greater than or equal to 2 fold (" $\geq 2x$ ") in 39% of the patients, greater than or equal to 2.5 fold in 36% of the patients, and greater than or equal to 5 fold in 27% of the patients examined.

Quantitative PCR of a number of normal tissues and tumor cell lines, particularly colorectal carcinoma cell lines was used to analyze expression of TTK. Quantitative real-time PCR was performed by first isolating RNA from cells using a Roche RNA Isolation kit according to manufacturer's directions. One microgram of RNA was used to synthesize a first-strand cDNA using MMLV reverse transcriptase (Ambion) using the manufacturers buffer and recommended concentrations of oligo dT, nucleotides, and Rnasin. This first-strand cDNA served as a template for quantitative real-time PCR using the Roche light-cycler as recommended in the machine manual. TTK was amplified with the forward primer CGGAATCAAGTCTTCTAGCT (SEQ ID NO:1) and reverse primer GGTGCTCAAAAGTTGGTATG (SEQ ID NO:2) PCR product was quantified based on the cycle at which the amplification entered the linear phase of amplification in comparison to an internal standard and using the software supplied by the manufacturer. Small differences in amounts or total template in the first-strand cDNA reaction were eliminated by normalizing to amount of actin amplified in a separate quantitative PCR reaction using the forward primer 5'-CGGGAAATCGTGCGTGACATTAAG-3' (SEQ ID NO:3) and the reverse primer: 5'-TGATCTCCTTCTGCATCCTGTCGG-3' (SEQ ID NO:4). The results for TTK mRNA levels in normal tissues are shown in Fig. 1; the results for TTK mRNA levels in tumor cell lines are shown in Fig. 2. A brief description of the cell lines analyzed is provided in the table below.

Cell Line	Tissue Source	Cell Line	Tissue Source
MDA-MB-231	Human breast; high metastatic potential (micromets in lung; adenocarcinoma; pleural effusion)	Caco-2	Human colorectal adenocarcinoma
MDA-MB-435	Human breast, high metastatic potential (macrometastases in lung)	SW620	Human colorectal adenocarcinoma; from metastatic site (lymph node)
MCF-7	Human breast; non-metastatic	LS174T	High metastatic potential human colorectal adenocarcinoma
MDA-MB-468	Human breast; adenocarcinoma	LOVO	Human colorectal adenocarcinoma; colon; from metastatic site (colon)
Alab	Human breast, metastatic	HT29	Human colorectal adenocarcinoma; colon
SKOV3	Human ovarian adenocarcinoma	SW480	Human colorectal adenocarcinoma; colon
OVCAR3	Human ovarian adenocarcinoma	HCT116	Human colorectal carcinoma; colon
KM12C	Human colon; low metastatic potential	Colo 320DN	Human colorectal adenocarcinoma; colon
KM12L4	Human colon; high metastatic potential (derived from Km12C)	T84	Human colorectal carcinoma; colon; from metastatic site (lung)
DU 145	Human prostate; carcinoma; from metastatic site: brain	HCT15	Human colorectal adenocarcinoma; colon
HT1080	Human sarcoma cell line;	CCD112	Human colorectal adenocarcinoma, low metastatic potential
HMVEC	Primary human microvascular endothelial cells	DLD1	Human colon; colorectal adenocarcinoma
184B5	normal breast epithelial cells; chemically transformed	293	kidney epithelial cells
LNCAP	prostate carcinoma; metastasis to left supraclavicular lymph	GRDP2	primary prostate epithelium
U373MG	glioblastoma cell	IMR90	primary lung fibroblast
WOCA	primary prostate epithelium	PC3	prostate cancer; androgen receptor negative

TTK was expressed in normal cells (Figure 1), with thymus and testis identified as the normal tissues that most highly express the gene for TTK. Numerous cancer cells, however,

displayed a significantly elevated level of TTK expression (Figure 2) as compared to most wild-type tissues.

### EXAMPLE 3: HIERARCHICAL CLUSTERING AND STRATIFICATION OF COLON CANCERS USING

#### DIFFERENTIAL EXPRESSION DATA

Differential expression patterns from Example 2 were analyzed by applying hierarchical clustering methods to the data sets (see Eisen *et al.* (1998) *PNAS* 95:14863-14868). In short, hierarchical clustering algorithms are based on the average-linkage method of Sokal and Michener (Sokal, RR & Michener, CD (1958) *Univ. Kans. Sci. Bull.* 38, 1409-1438), which was developed for clustering correlation matrixes. The object of this algorithm is to compute a dendrogram that assembles all elements into a single tree. For any set of *n* genes, an upper-diagonal similarity matrix is computed which contains similarity scores for all pairs of genes. The matrix is scanned to identify the highest value (representing a similar pair of genes). Using this technique, four groups of differential expression patterns were identified and assigned to clusters.

Application of hierarchical clustering to the data from Example 2 revealed that IGF2 (insulin-like growth factor 2), TTK (serine, threonine, tyrosine kinase implicated in the cell cycle), MAPKAPK2 (mitogen-activated protein (MAP) kinase-activated protein kinase), and MARCKS (myristoylated alanine-rich C kinase substrate, which is a substrate of protein kinase C) are concurrently upregulated as detected in 9 out of the 33 colon cancer patient samples examined. The data for these experiments is presented in graphical form in Figs. 3-6. The concurrent upregulation suggests that these genes are co-regulated and that patients with an elevated serum level of IGF2 may be candidates for treatment with inhibitors to TTK, MAPKAP kinase 2, MARCKS and/or IGF2.

### EXAMPLE 4: ANTISENSE REGULATION OF TTK EXPRESSION

Additional functional information on TTK was generated using antisense knockout technology. TTK expression in cancerous cells was further analyzed to confirm the role and function of the gene product in tumorigenesis, *e.g.*, in promoting a metastatic phenotype.

A number of different oligonucleotides complementary to TTK mRNA were designed as potential antisense oligonucleotides, and tested for their ability to suppress expression of

TTK. The ability of each designed antisense oligonucleotide to inhibit gene expression was tested through transfection into SW620 colon colorectal carcinoma cells. For each transfection mixture, a carrier molecule, preferably a lipitoid or cholesterol, was prepared to a working concentration of 0.5 mM in water, sonicated to yield a uniform solution, and filtered through a 0.45  $\mu$ m PVDF membrane. The antisense or control oligonucleotide was then prepared to a working concentration of 100  $\mu$ M in sterile Millipore water. The oligonucleotide was further diluted in OptiMEM™ (Gibco/BRL), in a microfuge tube, to 2  $\mu$ M, or approximately 20  $\mu$ g oligo/ml of OptiMEM™. In a separate microfuge tube, lipitoid or cholesterol, typically in the amount of about 1.5-2 nmol lipitoid/ $\mu$ g antisense oligonucleotide, was diluted into the same volume of OptiMEM™ used to dilute the oligonucleotide. The diluted antisense oligonucleotide was immediately added to the diluted lipitoid and mixed by pipetting up and down. Oligonucleotide was added to the cells to a final concentration of 30 nM.

The level of target mRNA (TTK) in the transfected cells was quantitated in the cancer cell lines using the Roche LightCycler™ real-time PCR machine. Values for the target mRNA were normalized versus an internal control (*e.g.*, beta-actin). For each 20  $\mu$ l reaction, extracted RNA (generally 0.2-1  $\mu$ g total) was placed into a sterile 0.5 or 1.5 ml microcentrifuge tube, and water was added to a total volume of 12.5  $\mu$ l. To each tube was added 7.5  $\mu$ l of a buffer/enzyme mixture, prepared by mixing (in the order listed) 2.5  $\mu$ l H<sub>2</sub>O, 2.0  $\mu$ l 10X reaction buffer, 10  $\mu$ l oligo dT (20 pmol), 1.0  $\mu$ l dNTP mix (10 mM each), 0.5  $\mu$ l RNAsin® (20u) (Ambion, Inc., Hialeah, FL), and 0.5  $\mu$ l MMLV reverse transcriptase (50u) (Ambion, Inc.). The contents were mixed by pipetting up and down, and the reaction mixture was incubated at 42°C for 1 hour. The contents of each tube were centrifuged prior to amplification.

An amplification mixture was prepared by mixing in the following order: 1X PCR buffer II, 3 mM MgCl<sub>2</sub>, 140  $\mu$ M each dNTP, 0.175 pmol each oligo, 1:50,000 dil of SYBR® Green, 0.25 mg/ml BSA, 1 unit *Taq* polymerase, and H<sub>2</sub>O to 20  $\mu$ l. (PCR buffer II is available in 10X concentration from Perkin-Elmer, Norwalk, CT). In 1X concentration it contains 10 mM Tris pH 8.3 and 50 mM KCl. SYBR® Green (Molecular Probes, Eugene, OR) is a dye which fluoresces when bound to double stranded DNA. As double stranded PCR product is produced during amplification, the fluorescence from SYBR® Green increases. To each 20  $\mu$ l aliquot of amplification mixture, 2  $\mu$ l of template RT was added, and amplification was carried

out according to standard protocols.

The following antisense oligonucleotides were shown to effectively deplete TTK RNA in the transfection assays:

Oligo 79-5AS: GGGACTCTTCCAAATGGGCATGACT (SEQ ID NO:5)

5 Oligo 79-9AS: TCCAGTAACTCTTGCGTTCCCATGG (SEQ ID NO:6)

The reverse control of each of these antisense oligonucleotides were synthesized, as were oligonucleotides with the identical sequence of the antisense oligonucleotides in reverse orientation (Reverse Control):

Oligo 79-5RC: TCAGTACGGGTAAACCTTCTCAGGG (SEQ ID NO:7)

10 Oligo 79-9RC: GGTACCCTTGCGTTCTCAATGACCT (SEQ ID NO:8)

The antisense oligonucleotides were introduced into a test cell and the effect upon TTK expression of the corresponding gene, as well as the effect induction of the cancerous phenotype, was examined as described below.

#### 15 EXAMPLE 5: EFFECT OF TTK EXPRESSION ON PROLIFERATION

The effect of TTK on proliferation was assessed in metastatic breast cancer cell lines (MDA-MB-231 ("231")), SW620 colon colorectal carcinoma cells, or 847 human immortal fibroblast cells. Transfection was carried out as described above in Example 4.

20 Cells were plated to approximately 60-80% confluency in 96-well dishes. Antisense or reverse control oligonucleotide was diluted to 2  $\mu$ M in OptiMEM™ and added to OptiMEM™ into which the delivery vehicle, lipitoid 116-6 in the case of SW620 cells or 1:1 lipitoid 1:cholesteroid 1 in the case of MDA-MB-231 cells, had been diluted. The oligo/ delivery vehicle mixture was then further diluted into medium with serum on the cells. The final concentration of oligonucleotide for all experiments was 300 nM, and the final ratio of oligo to  
25 delivery vehicle for all experiments was 1.5 nmol lipitoid/ $\mu$ g oligonucleotide. Cells were transfected overnight at 37°C and the transfection mixture was replaced with fresh medium the next morning.

30 Transfection of the antisense oligonucleotides into both SW620 colorectal carcinoma cells (Fig. 7) and 231 cells (Fig. 8) resulted in a decreased rate of proliferation compared to matched reverse control (RC) and oligonucleotides, but no inhibition of growth of 847 human

immortal fibroblast cells (Fig. 11), suggesting possible tissue or transformation specificity in the functional role for the TTK protein.

#### EXAMPLE 6: EFFECT OF TTK EXPRESSION ON COLONY FORMATION

5           The effect of TTK expression upon colony formation was tested in a soft agar assay. Soft agar assays were conducted by first establishing a bottom layer of 2 ml of 0.6% agar in media plated fresh within a few hours of layering on the cells. The cell layer was formed on the bottom layer by removing cells transfected as described above from plates using 0.05% trypsin and washing twice in media. The cells were counted in a Coulter counter, and  
10           resuspended to  $10^6$  per ml in media. 10  $\mu$ l aliquots are placed with media in 96-well plates (to check counting with WST1), or diluted further for soft agar assay. 2000 cells are plated in 800  $\mu$ l 0.4% agar in duplicate wells above 0.6% agar bottom layer. After the cell layer agar solidifies, 2 ml of media is dribbled on top and antisense or reverse control oligo is added without delivery vehicles. Fresh media and oligos are added every 3-4 days. Colonies are  
15           formed in 10 days to 3 weeks. Fields of colonies were counted by eye. Wst-1 metabolism values can be used to compensate for small differences in starting cell number. Larger fields can be scanned for visual record of differences.

As shown in Fig. 9, antisense oligonucleotides to TTK (79-9AS) led to decreased colony size and number compared to control reverse control oligonucleotides (79-9RC) or to  
20           control oligonucleotides (52-3AS: TAGGTCTTTGGCCGGTGATGGGTCG (SEQ ID NO:9) and 52-3RC: GCTGGGTAGTGGCCGGTTTCTGGAT (SEQ ID NO:10)). The 52-3 antisense oligonucleotide is directed to the hD53 mRNA, and serves as a negative control in the experiment.

#### EXAMPLE 7: INDUCTION OF CELL DEATH UPON DEPLETION OF TTK ("ANTISENSE KNOCKOUT")

SW620 cells were transfected as described for proliferation assays. For cytotoxic effect in the presence of cisplatin (cis), the same protocol was followed but cells were left in the presence of 2  $\mu$ M drug. Each day, cytotoxicity was monitored by measuring the amount of  
30           LDH enzyme released in the medium due to membrane damage. The activity of LDH was measured using the Cytotoxicity Detection Kit from Roche Molecular Biochemicals. The data

is provided as a ratio of LDH released in the medium vs. the total LDH present in the well at the same time point and treatment (rLDH/tLDH). A positive control using antisense and reverse control oligonucleotides for BCL2 (a known anti-apoptotic gene) shows that loss of message for BCL2 leads to an increase in cell death compared with treatment with the control oligonucleotide (background cytotoxicity due to transfection).

The following antisense oligonucleotides were tested for the ability to deplete the message levels of the gene corresponding to the indicated cluster. Oligo Name : AS or RC provides the name of the target gene or name of the oligo, and whether the oligo is antisense (AS) or a reverse control (RC).

Oligo Name: Antisense (AS) or Reverse Control (RC)	Oligo Sequence	SEQ ID NO:
Chir39-5:AS	ACTCATCTGGCTGGGCTATGGTGGT	SEQ ID NO:11
Chir39-5:RC	TGGTGGTATCGGGTCGGTCTACTCA	SEQ ID NO:12
Chir79-9:AS	TCCAGTAACTCTTGCGTTCCCATGG	SEQ ID NO:6
Chir79-9:RC	GGTACCCTTGCGTTCTCAATGACCT	SEQ ID NO:8

As shown in Fig. 12, Chiron 79-9 (TTK) antisense does not sensitize the cells to treatment by cisplatin at a detectable level, but leads to increased death compared to control oligo at day 3.

#### EXAMPLE 8: SAMPLE ASSAY FOR AGENTS THAT MODULATE TTK ACTIVITY

This assay may be performed in microtitre plates. TTK was purified as a 6x His tagged fusion protein using a baculovirus expression system. Essentially 20 ul of 20 nM TTK (100k Da) in TTK kinase buffer comprising 50 mM Hepes pH 7.4, 2mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, 1 mM NaF, 50mM NaCl, 1 mM DTT and 1 mg/ml BSA was added to 5ul of a candidate agent diluted in 20% DMSO, 10 ul of a 2.8 uM solution of a biotinylated substrate peptide derived from cdc25, such as Biotin-SGSGSGLYRSPSPENLNRPR-NH<sub>2</sub> (SEQ ID NO:27) or Biotin-GGGGLYRSPSPENLNRK-OH (SEQ ID NO:28) and 5 ul of 80 nM <sup>33</sup>P-γATP in a well of a microtitre plate. Samples were mixed, incubated for 2 hours and each reaction is terminated using 20 ul of 0.5 M EDTA pH 8.0. 50 ul of the sample is transferred to a 96 well flat bottom Streptavidin coated flash plate, and the sample is incubated with the plate for 1hr at room temperature. The wells of the plate are washed four times with 250 ul of calcium and



magnesium-free phosphate buffered saline, and scintillation fluid is added to the sample. Activity of TTK was measured by calculating the emission of  $^{33}\text{P}$ , transferred by TTK from  $^{33}\text{P}$ - $\gamma\text{ATP}$  to a substrate peptide, by scintillation.

Agents modulating TTK activity can be identified by comparing the activity of TTK in the presence of a candidate agent to the activity of TTK in the absence of a candidate agent.

While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the invention.